



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12P 1/00, 1/02, 7/40, C07C 53/00, C12N 1/14</b>		A1	(11) International Publication Number: <b>WO 99/61645</b> (43) International Publication Date: 2 December 1999 (02.12.99)		
(21) International Application Number: PCT/JP99/02707		(74) Agent: SEKI, Hideo; Fujisawa Pharmaceutical Co., Ltd., Osaka Factory, 1-6, Kashima 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532-8514 (JP).			
(22) International Filing Date: 21 May 1999 (21.05.99)		(81) Designated States: BR, CA, CN, JP, KR, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).			
(30) Priority Data: PP 3771 28 May 1998 (28.05.98) AU		Published <i>With international search report.</i>			
(71) Applicant (for all designated States except US): FUJI-SAWA PHARMACEUTICAL CO., LTD. [JP/JP]; 4-7, Doshomachi 3-chome, Chuo-ku, Osaka-shi, Osaka 541-8514 (JP).					
(72) Inventors; and					
(75) Inventors/Applicants (for US only): OHTSU, Yoshihiro [JP/JP]; 3762-13, Shin-ishige, Ishigemachi, Yuki-gun, Ibaraki 300-2706 (JP). TANAKA, Miho [JP/JP]; 6-5-5-306, Nakamuraminami, Tsuchiura-shi, Ibaraki 300-0843 (JP). SHIBATA, Toshihiro [JP/JP]; 4-12-3, Yayoi-gaoka, Sanda-shi, Hyogo 669-1546 (JP). SAKAMOTO, Kazutoshi [JP/JP]; 2-17-14, Ottominami, Tsuchiura-shi, Ibaraki 300-0845 (JP). TSURUMI, Yasuhisa [JP/JP]; 3-19-1-3-301, Azuma, Tsukuba-shi, Ibaraki 305-0031 (JP). TAKASE, Shigehiro [JP/JP]; 1-12-10, Sousha, Ishioka-shi, Ibaraki 315-0016 (JP). HINO, Motohiro [JP/JP]; 13-3-1003, Tozaki-cho, Tsuchiura-shi, Ibaraki 300-0031 (JP).					
(54) Title: NOVEL COMPOUND, WF00144					
(57) Abstract					
<p>The present invention provides a new bioactive compound, WF00144 substance or its salt which has an inhibitory activity against gluconeogenesis, and a process for production of the same, which comprises culturing a WF00144 substance-producing strain belonging to the genus <i>Phoma</i> in a nutrient medium and recovering the WF00144 substance. Also provided are a pharmaceutical composition containing the WF00144 substance or pharmaceutically acceptable salt thereof, a use of the WF00144 substance as a medicament and use of the WF00144 substance for the manufacture of a medicament for therapeutic treatment or prevention of diabetes in human or animal.</p>					

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

<b>AL</b>	Albania	<b>ES</b>	Spain	<b>LS</b>	Lesotho	<b>SI</b>	Slovenia
<b>AM</b>	Armenia	<b>FI</b>	Finland	<b>LT</b>	Lithuania	<b>SK</b>	Slovakia
<b>AT</b>	Austria	<b>FR</b>	France	<b>LU</b>	Luxembourg	<b>SN</b>	Senegal
<b>AU</b>	Australia	<b>GA</b>	Gabon	<b>LV</b>	Latvia	<b>SZ</b>	Swaziland
<b>AZ</b>	Azerbaijan	<b>GB</b>	United Kingdom	<b>MC</b>	Monaco	<b>TD</b>	Chad
<b>BA</b>	Bosnia and Herzegovina	<b>GE</b>	Georgia	<b>MD</b>	Republic of Moldova	<b>TG</b>	Togo
<b>BB</b>	Barbados	<b>GH</b>	Ghana	<b>MG</b>	Madagascar	<b>TJ</b>	Tajikistan
<b>BE</b>	Belgium	<b>GN</b>	Guinea	<b>MK</b>	The former Yugoslav	<b>TM</b>	Turkmenistan
<b>BF</b>	Burkina Faso	<b>GR</b>	Greece		Republic of Macedonia	<b>TR</b>	Turkey
<b>BG</b>	Bulgaria	<b>HU</b>	Hungary	<b>ML</b>	Mali	<b>TT</b>	Trinidad and Tobago
<b>BJ</b>	Benin	<b>IE</b>	Ireland	<b>MN</b>	Mongolia	<b>UA</b>	Ukraine
<b>BR</b>	Brazil	<b>IL</b>	Israel	<b>MR</b>	Mauritania	<b>UG</b>	Uganda
<b>BY</b>	Belarus	<b>IS</b>	Iceland	<b>MW</b>	Malawi	<b>US</b>	United States of America
<b>CA</b>	Canada	<b>IT</b>	Italy	<b>MX</b>	Mexico	<b>UZ</b>	Uzbekistan
<b>CF</b>	Central African Republic	<b>JP</b>	Japan	<b>NE</b>	Niger	<b>VN</b>	Viet Nam
<b>CG</b>	Congo	<b>KE</b>	Kenya	<b>NL</b>	Netherlands	<b>YU</b>	Yugoslavia
<b>CH</b>	Switzerland	<b>KG</b>	Kyrgyzstan	<b>NO</b>	Norway	<b>ZW</b>	Zimbabwe
<b>CI</b>	Côte d'Ivoire	<b>KP</b>	Democratic People's	<b>NZ</b>	New Zealand		
<b>CM</b>	Cameroon		Republic of Korea	<b>PL</b>	Poland		
<b>CN</b>	China	<b>KR</b>	Republic of Korea	<b>PT</b>	Portugal		
<b>CU</b>	Cuba	<b>KZ</b>	Kazakhstan	<b>RO</b>	Romania		
<b>CZ</b>	Czech Republic	<b>LC</b>	Saint Lucia	<b>RU</b>	Russian Federation		
<b>DE</b>	Germany	<b>LI</b>	Liechtenstein	<b>SD</b>	Sudan		
<b>DK</b>	Denmark	<b>LK</b>	Sri Lanka	<b>SE</b>	Sweden		
<b>EE</b>	Estonia	<b>LR</b>	Liberia	<b>SG</b>	Singapore		

## DESCRIPTION

## NOVEL COMPOUND, WF00144

## 5 TECHNICAL FIELD

The present invention relates to a new bioactive compound, hereinafter entitled WF00144 substance or its salt which is useful as a medicament.

## 10 DISCLOSURE OF INVENTION

The present invention relates to a new bioactive compound, WF00144 substance or its salt.

15 More particularly, it relates to a novel compound, WF00144 substance or its salt which has an inhibitory activity against gluconeogenesis, to a process for preparation thereof, to a pharmaceutical composition comprising the same, which is useful as antidiabetic agents, and to a use thereof as a medicament.

20 Accordingly, one object of this invention is to provide a novel compound, WF00144 substance which is of use for treating and preventing diabetes, and the like.

25 Another object of this invention is to provide a process for production of the WF00144 substance by fermentation of a WF00144 substance-producing microorganism in a nutrient medium.

30 A further object of this invention is to provide a pharmaceutical composition containing, as an active ingredient, the WF00144 substance.

35 Still further object of this invention is to provide a use of the WF00144 substance for treating and preventing diabetes and the like.

The WF00144 substance can be produced by fermentation of the WF00144 substance-producing microorganism, especially, strain belonging to

the genus *Phoma* such as *Phoma* sp. No. 00144 in a nutrient medium.

It is to be understood that the production of the WF00144 substance is not limited to the use of the particular organism described herein, which is given for the illustrative purpose only. This invention also includes the use of any mutants which are capable of producing the WF00144 substance including natural mutants as well as artificial mutants which can be produced from the described organism by conventional means such as irradiation of X-ray, ultra-violet radiation, genetic engineering treatment with N-methyl-N'-nitro-N-nitrosoguanidine, 2-aminopurine, and the like.

#### Characteristics of producing strain No.00144

The fungal strain No.00144 was originally isolated from a decayed leaf sample, collected at Ashiwada-mura, Minamitsuru-gun, Yamanashi-ken, Japan. This organism grew restrictedly on various culture media, and formed olive brown to dark green colonies. On or in the agar media, the strain produced pycnidial conidiomata. The conidiomata were globose to subglobose, brown, and formed ampulliform conidiogenous cells on their inner walls. Conidia were hyaline, one-celled and globose to subglobose. The strain did not form teleomorph. Its mycological characteristics were as follows.

Cultural characteristics on various agar media are summarized in Table 1. Culture on potato dextrose agar grew restrictedly, attaining 2.0-3.0 cm in diameter two weeks later at 25°C. This colony surface was plane to raised, felty to cottony, and greenish gray to dark green. Many conidiomata were formed on or in the media. The reverse color was dark gray to reddish gray, and sometimes producing reddish soluble pigments. Colonies on corn meal agar spread more restrictedly than on potato dextrose agar, attaining 1.5-2.5 cm in diameter under the same conditions. The surface was plane, felty, exudate, brownish gray to olive brown at the center, and dark gray to dark green at the margin. The reverse was dark gray to dark green. Conidiomata were abundantly formed.

The morphological characteristics were determined from the cultures on a Miura's LCA plate (Miura, K. and M. Kudo: *Trans. Mycol. Soc. Japan*, 11:116-118, 1970). Conidiomata were pycnidial, superficial or immersed,

separate and brown to dark brown. Their shape was globose to subglobose, sometimes papillate, distinctly ostiolate, unilocular, and 60-90(-110) x 55-85  $\mu\text{m}$  in size. Ostioles were 10-30(-35)  $\mu\text{m}$  in diameter. In old culture, a few pycnidia formed 1-3(-5) setae around the ostiole. The setae were dark brown, smooth, thick-walled, unbranched, somewhat flexuous, acute at the apex, and 15-24 x 4-6  $\mu\text{m}$ . Pycnidial walls were thin and composed of 1-2 cells layer. The cells of pycnidial walls were thick-walled, brown, irregularly shaped, 3.5-8 x 2.5-6.5  $\mu\text{m}$ , and formed textura angularis. The inner pycnidial walls formed directly conidiogenous cells without conidiophores. The conidiogenous cells were discrete, acrogenous, hyaline, smooth, ampulliform to lageniform, and 3.5-8 x 2.5-6  $\mu\text{m}$ . The tips of conidiogenous cells were 1.5-2.5  $\mu\text{m}$  wide. Conidia were enteroblastic, phialidic, hyaline, smooth, one-celled, globose to subglobose, with a small projection at the base, and 2.5-3.5 x (2-)2.5-3  $\mu\text{m}$ . Vegetative hyphae were smooth, septate, brown and branched. The hyphal cells were cylindrical and 1.5-5  $\mu\text{m}$  in width. Chlamydospores were not observed.

Strain No.00144 was able to grow at the temperature range from 5 to 30°C, with the growth optimum at 21 to 24°C. These temperature data were determined on potato dextrose agar (made by NISSUI).

On the basis of comparing the morphological characteristics with fungal taxonomic criteria by von Arx (J. A. von Arx: The Genera of Fungi - Sporulating in Pure Culture. 3rd ed., pp.315, J. Cramer, Vaduz, 1974) and by Sutton (B. C. Sutton: The Coelomycetes - Fungi Imperfecti with Pycnidia, Acervuli and Stroma., pp.696, Commonwealth Mycological Institute, Kew, 1980.), strain No.00144 was considered to belong to the coelomycete genus *Phoma* Sacc. 1880 (Sphaeropsidales). Thus, we identified this isolate as one strain of the genus *Phoma*, and named it *Phoma* sp. No.00144. The strain has been deposited to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, at 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan, as FERM BP-6360 (deposited date : May 19, 1998) under the Budapest Treaty.

Table 1. Cultural characteristics of strain No.00144.

Media	Cultural characteristics
5 Malt extract agar*	G:Rather restrictedly, 2.5-3.5 cm S: Circular, plane, felty, radiately sulcate, sometimes exudate, formed some pycnidia, dark green (28F3-28F4) to dull green (28D3-28E3)
10	R:Grayish brown (8F3) to dark brown (8F4-8F5), sometimes producing reddish soluble pigments
15 Potato dextrose agar (Difco 0013)	G:Restrictedly, 2.0-3.0 cm S: Circular, plane to raised, felty to cottony, formed many pycnidia, greenish gray (27F2) to dark green (27F3)
20 Czapek's solution agar*	R:Dark gray (1F1) to reddish gray (12F2), sometimes producing reddish soluble pigments G:Restrictedly, 2.0-3.0 cm S: Circular, plane or somewhat raised, felty, radiately sulcate or wrinkly, exudate, formed pycnidia abundantly, olive (1F3-1E4) to olive gray (1F2), and reddish brown (8E3-8E5) at the margin
25	R:Dark brown (8F3-8F4), and light brown (7D4) to brown (7E4) at the margin, sometimes producing orange soluble pigments
30 Sabouraud dextrose agar (Difco 0190)	G:Restrictedly, 2.0-3.0 cm S: Circular, plane to centrally raised, felty, radiately sulcate, formed no pycnidia, dull green (28E4) to dark green (28F3), and greenish gray (28D2) at the margin
35	R:Dark ruby (12F3) to dark purple (14F3),

		sometimes producing reddish soluble pigments
5	Emerson Yp Ss agar (Difco 0739)	G:Very restrictedly, 1.0-2.0 cm S: Circular, plane, felty, radiately sulcate, formed pycnidia abundantly, dark gray (1F1) R:Dark gray (1F1) to dark blue (22F4)
10	Corn meal agar (Difco 0386)	G:Restrictedly, 1.5-2.5 cm S: Circular, plane, felty, exudate, abundantly formed pycnidia, brownish gray (4F2) to olive brown (4F3) at the center, and dark gray (1F1) to dark green (28F4) at the margin R:Dark gray (1F1) to dark green (28F4)
15	MY20 agar*	G:Restrictedly, 1.5-2.5 cm S: Circular, plane, felty, sometimes funiculose, radiately sulcate or wrinkly, formed no pycnidia, dull green (28D3-28E3) or brownish gray (4E2) to olive brown (4E3) R:Grayish brown (7F3) to dark brown (7F4), and light brown (7D4) to brown (7E4) at the margin, sometimes producing pale orange soluble pigments
20	Oatmeal agar (Difco 0552)	G:Very restrictedly, 1.0-2.0 cm S: Circular, plane, felty, exudate, abundantly formed pycnidia, brownish gray (4D2-4F2) to olive brown (4F3) at the center, and dark gray (1F1) at the margin

30 Abbreviation G: growth, measuring colony size in diameter, S: colony surface, R: reverse.

35 \*: The compositions of malt extract agar, Czapek's solution agar and MY20 agar were based on JCM Catalogue of Strains (Nakase, T., 6th ed., pp.617, Japan Collection of Microorganisms, the Institute of Physical and Chemical Research, Saitama, 1995).

These characteristics were observed after 14 days of incubation at 25°C. The color descriptions were based on Methuen Handbook of Colour (Kornerup, A. and J. H. Wanscher, 3rd ed., pp.252, Methuen, London, 1978).

5 Production of the WF00144 substance

The WF00144 substance is produced when the WF00144 substance-producing strain belonging to the genus *Phoma* is grown in a nutrient medium containing sources of assimilable carbon and nitrogen under aerobic 10 conditions (e. g. shaking culture, submerged culture, etc.).

The preferred sources of carbon in the nutrient medium are carbohydrates such as glucose, sucrose, starch, fructose, glycerin, or the like.

The preferred sources of nitrogen are peanut powder, yeast extract, peptone, gluten meal, cotton seed flour, soybean powder, soybean meal, corn 15 steep liquor, dried yeast, wheat germ, etc., as well as inorganic and organic nitrogen compounds such as ammonium salts (e. g. ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.), urea or amino acid, or the like.

The carbon and nitrogen sources, though advantageously employed in 20 combination, need not to be used in their pure form because less pure materials, which contain traces of growth factors and considerable quantities of mineral nutrients, are also suitable for use.

When desired, there may be added to the medium mineral salts such as sodium or calcium carbonate, sodium or potassium phosphate, sodium or 25 potassium chloride, sodium or potassium iodide, magnesium salts, copper salts, zinc salts, iron salts, or cobalt salts, or the like.

If necessary, especially when the culture medium foams seriously a defoaming agent, such as liquid paraffin, fatty oil, plant oil, mineral oil or silicone, or the like may be added.

Agitation and aeration of the culture mixture may be accomplished in a 30 variety of ways, such as agitation by a propeller or similar mechanical agitation equipment, by revolving or shaking the fermenter, and the like.

The fermentation is usually conducted at a temperature between about 35 10°C and 40°C, preferably 20°C to 35°C, for a period of about 24 hours to 120 hours, which may be varied according to fermentation conditions and

scales.

When the fermentation is completed, the culture broth is then subjected for recovery of the WF00144 substance to various procedures conventionally used for recovery and purification of biological active substance, for instance, solvent extraction with an appropriate solvent or a mixture of some solvents, chromatography or recrystallization from an appropriate solvent or a mixture thereof.

The WF00144 substance obtained can be converted to its salt in a conventional manner. The salt of the WF00144 substance may include a salt with an organic or inorganic base such as alkaline metal salt (e.g. sodium or potassium salt ), alkaline earth metal salt (e.g. calcium or magnesium salt ), organic amine salt (e.g. ethanolamine salt, etc.) amino acid salt (e.g. arginine salt, lysine salt, histidine salt, etc.) and the like.

The WF00144 substance as obtained has the following physico-chemical properties:

(1) Appearance:

white powder

(2) Molecular formula :

$C_{27}H_{40}O_9$

(3) Elementary Analysis:

Calcd for  $C_{27}H_{40}O_9 \cdot 1/2H_2O$

C 62.65, H 7.98

25 Found:

C 62.22, H 7.97

(4) Molecular weight:

ESI-MS(negative): m/z 507 ( $M - H$ )<sup>-</sup>

(Calcd. Molecular weight: 508.61)

30 (5) Melting point:

85.89 °C (dec)

(6) Optical rotation:

$[\alpha]D(23^\circ C) = -16^\circ$  (c=0.2, in chloroform)

(7) Ultraviolet absorption spectrum:

$\lambda_{max}$  (methanol): 275 nm ( $\epsilon=8000$ )

## (8) Solubility:

Soluble: acetonitrile, chloroform, ethyl acetate, dimethylsulfoxide

Slightly soluble: n-hexane

Insoluble: water

## (9) Color reaction:

Positive: cerium sulfate reaction, iodine vapor reaction,

Negative: Molish's reaction, ninhydrin reaction, Dragendorff reaction,  
Ehrlich's reaction

## (10) Thin layer chromatography (TLC):

Stationary phase	Developing solvent	Rf value
Silica Gel 60 F254*	n-hexane : ethyl acetate : acetic acid ( 50: 50: 1, v/v )	0.25

\* made by E. Merck

## (11) High Performance Liquid Chromatography (HPLC):

Condition:

Column: YMC-Pack Pro C18 AS-302 \*\*(4.6 mmφ x 150mmL)

Mobile phase: 50% aqueous acetonitrile containing 0.05%  
trifluoroacetic acid

Flow rate: 1 ml/min

Detection: UV at 280 nm

Retention time: 7.9 min

\*\*: made by YMC Co., Ltd.

## (12) Infrared absorption spectrum:

vmax (KBr): 3480, 2980, 2930, 1730, 1710, 1650, 1620, 1460,  
1380, 1160, 1140 cm<sup>-1</sup>

(13) <sup>1</sup>H Nuclear magnetic resonance spectrum:

(500 MHz, CD<sub>3</sub>CN) δ(ppm):

15.0 (1H, br s), 7.76 (1H, d, 5), 5.97 (1H, d, 5), 5.74 (1H, br s),  
4.22 (1H, dd, 10.5, 9.5), 4.13 (1H, dd, 10.5, 4.5), 3.20 - 3.13 (2H, m),  
2.63 (1H, m), 2.53 (1H, dd, 13, 10), 2.18 (1H, m), 2.12 (3H, br s), 2.02  
(1H, m), 1.85 (1H, s), 1.80 (1H, m), 1.53 (3H, s), 1.57 - 1.47 (2H, m),  
1.37 (1H, m), 1.27 (1H, m), 1.27 (3H, s), 1.16 (3H, s), 1.12 (1H, dd,

12, 14), 0.82 (3H, t, 7), 0.68 (3H, d, 6.5).

5 (14)  $^{13}\text{C}$  Nuclear magnetic resonance spectrum:

(125 MHz, CDCl<sub>3</sub>) δ(ppm):

217.4 (s), 209.3 (s), 173.5 (d), 170.9 (s), 167.5 (s), 153.5 (s),  
119.7 (d), 102.7 (d), 78.1 (s), 69.6 (t), 68.9 (s), 50.5 (d), 50.2 (s),  
48.4 (t), 46.4 (t), 45.7 (d), 41.9 (t), 41.5 (d), 41.3 (d), 31.0 (q),  
30.1 (d), 24.6 (q), 22.5 (t), 21.3 (q), 20.6 (q), 19.1 (q), 13.4 (q).

10 (15) Nature of substance:

acidic substance.

#### 15 Biological properties of the WF00144 substance

The WF00144 substance possesses pharmacological activities such as the inhibitory activity against gluconeogenesis, and the like, and therefore are useful for the treatment and prevention of diabetes, and the like.

20 And further, the WF00144 substance may be useful for various diseases because of its useful pharmaceutical activity such as an inhibitory activity against gluconeogenesis, and so on.

25 As examples for showing biological activities of the WF00144 substance, some biological data are explained in the following.

#### 25 Test (Effect of WF00144 substance on rat hepatocytes gluconeogenesis)

30 Hepatocytes were prepared from 24 hours starved male Wistar rat (150-200g) by the collagenase perfusion technique. Cells were cultured in William's E medium containing 5%(v/v) fetal bovine serum, 0.1mg/ml kanamycin for 6 hours at 96-well tissue culture plates. Cells were washed with phosphate-buffered saline and incubated with Dulbecco's Modified Eagle's Medium without glucose, supplemented with 20mM pyruvate, 1x10<sup>-7</sup> M glucagon, 0.1mg/ml kanamycin and 1%(v/v) fetal bovine serum. After 15 hours, glucose produced into the medium was determined by enzymatically method. Gluconeogenesis rate was performed as glucose value derived from 35 pyruvate.

The half-maximal inhibitory concentration of WF00144 substance on rat hepatocytes gluconeogenesis was  $0.08\mu\text{g}/\text{ml}$ .

The pharmaceutical composition of this invention can be used in the form of pharmaceutical preparation, for example, in solid, semisolid or liquid form, which contains the WF00144 substance or its pharmaceutically acceptable salt, as an active ingredient in admixture with an organic or inorganic carrier or excipient suitable for external, enteral or parenteral administrations. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, injections, ointments, liniments, eye drops, lotion, gel, cream, and any other form suitable for use.

The carriers which can be used are water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form, and in addition auxiliary, stabilizing, thickening, solubilizing and coloring agents and perfumes may be used.

For applying the above pharmaceutical composition to a patient including human beings and animals suffered from diabetes, it is preferable to apply it by intravenous, intramuscular, topical or oral administration. While the dosage of therapeutically effective amount of the WF00144 substance varies from and also depends upon the age and condition of each individual patient to be treated, the optimal dosage for the treatment of the patient suffered from diabetes may be selected from the range of 0.01 - 50 mg of the WF00144 substance per kg weight of the patient.

The following examples are given for the purpose of illustrating the present invention.

Example 1:

(1) Fermentation of *Phoma* sp. No.00144 for the production of the WF00144 substance

5 An aqueous seed medium (30ml) containing sucrose 4%, glucose 1%,  
soluble starch 2%, cotton seed meal 3%, soybean flour 1.5%,  $\text{KH}_2\text{PO}_4$  1%,  
 $\text{CaCO}_3$  0.2% was placed in a 100-ml Erlenmeyer flask and was sterilized at  
121°C for 30 minutes. A loopful of a slant culture of *Phoma* sp. No.00144 was  
inoculated in a seed flask. The inoculated flask was shaken on a rotary shaker  
10 (220 rpm, 5.1 cm throw) at 25°C for 4 days, and 3.2ml of the seed culture was  
transferred to 160-ml of the same sterile seed medium in the 500 ml  
Erlenmeyer flasks. The flasks were shaken on a rotary shaker (220rpm, 5.1 cm  
throw) at 25°C for 4 days, and 480 ml (three flasks) of second seed culture  
15 was inoculated to 20 liters of sterile production medium containing of glucose  
1%, starch acid hydrolysates 3%, wheat germ 1%,  $\text{KH}_2\text{PO}_4$  1%, Adecanol LG-  
109 (deforming agent, Asahi Denka Co.,Ltd.) 0.05%, Silicone KM-70  
(deforming agent, Shin-Etsu Chemical Co.,Ltd.) 0.05% in a 30-liter jar  
fermentor. Fermentation was carried out at 25°C for 7 days under aeration of  
20 liters / minute and agitation of 400rpm.

The production of the WF00144 substance in the fermentation broth was  
monitored by HPLC analysis indicated below.

20 Analytical condition:

Column; YMC-Pack Pro C18 AS-302 (4.6 mmφ x 150mmL, made by YMC  
Co., Ltd.)

Mobile phase; 50% aqueous acetonitrile containing 0.05% trifluoroacetic acid

Flow rate; 1ml/min

Detection; UV at 280nm

25 Retention time: 7.9min.

## (2) Isolation of WF00144 substance

30 The cultured broth (20L; containing 1.8g of WF00144 substance) was  
filtered with an aid of diatomaceous earth. The filtered mycelium was  
extracted with 20L of acetone by intermittent mixing for 1 hr. The acetone  
extract was filtered and diluted with twice volume of deionized water. The  
diluted filtrate was passed through a column (2L) of Diaion HP-20  
35 (Mitsubishi Chemical Co.,Ltd.). The column was washed with 50% aqueous  
methanol and eluted with 80% methanol. The eluate (4L) was concentrated in

vacuo to one liter and added with 3 liters of 0.07% aqueous trifluoroacetic acid, and then applied on a column (2L) of YMC GEL ODS-AM 120-S-50 (YMC Co.,Ltd.) packed with 25% aqueous acetonitrile containing 0.05% trifluoroacetic acid. The column was washed with 30% acetonitrile containing 0.05% trifluoroacetic acid, 40% acetonitrile containing 0.05% trifluoroacetic acid and eluted with 50% acetonitrile containing 0.05% trifluoroacetic acid. The fractions containing the WF00144 substance were combined and applied on a column (1L) of YMC GEL ODS-AM 120-S-50 (YMC Co.,Ltd.) packed with 25% aqueous acetonitrile containing 0.05% trifluoroacetic acid. The column was washed with 30% acetonitrile containing 0.05% trifluoroacetic acid, 40% acetonitrile containing 0.05% trifluoroacetic acid and eluted with 50% acetonitrile containing 0.05% trifluoroacetic acid. The fractions containing the WF00144 substance were combined and concentrated in vacuo to give residual water. The residual water was twice extracted with equal volume of ethyl acetate. The extract was concentrated in vacuo to small volume and added with several volumes of n-hexane, and then concentrated in vacuo to give 810mg of crude WF00144 substance as a powder.

The 60mg of crude WF00144 powder was dissolved in acetonitrile (0.6ml) and subjected to preparative HPLC, YMC-packed column (ODS-AM SH-343-5AM S-5 (20 mmφ x 250mmL; YMC Co.,Ltd.) with 50% aqueous acetonitrile containing 0.05% trifluoroacetic acid as a mobile phase and flow rate of 9.9 ml/min. Elution was monitored by analytical HPLC indicated below. The portion corresponding to the purified WF00144 substance was concentrated in vacuo to give residual water. This residue was twice extracted with equal volume of ethyl acetate and concentrated in vacuo to small volume. The concentrated extract was added with several volumes of n-hexane and dried in vacuo to give 36mg of purified WF00144 substance as a white powder.

Analytical condition:

Column; YMC-Pack Pro C18 AS-302 (4.6 mmφ x 150mmL)

Mobile phase; 50% aqueous acetonitrile containing 0.05% trifluoroacetic acid

Flow rate; 1ml/min

Detection; UV at 280nm

Retention time ; 7.9min.

## CLAIMS

1. WF00144 substance of the following physico-chemical properties or its salt:

5

(1) Molecular formula :



(2) Elementary Analysis:

Calcd for  $\text{C}_{27}\text{H}_{40}\text{O}_9 \cdot 1/2\text{H}_2\text{O}$

10

C 62.65, H 7.98

Found:

C 62.22, H 7.97

(3) Molecular weight:

ESI-MS(negative): m/z 507 (M-H)<sup>-</sup>

15

(Calcd. Molecular weight: 508.61)

(4) Melting point:

85-89 °C (dec)

20

(5) Optical rotation:

$[\alpha]_D(23^\circ\text{C}) = -16^\circ$  (c=0.2, in chloroform)

(6) Ultraviolet absorption spectrum:

$\lambda_{\text{max}}$  (methanol): 275 nm ( $\epsilon=8000$ )

25

(7) Solubility:

Soluble: acetonitrile, chloroform, ethyl acetate, dimethylsulfoxide

Slightly soluble: n-hexane

Insoluble: water

(8) Color reaction:

Positive: cerium sulfate reaction, iodine vapor reaction,

Negative: Molish's reaction, ninhydrin reaction, Dragendorff reaction,

Ehrlich's reaction

30

(9) Infrared absorption spectrum:

$\nu_{\text{max}}$  (KBr): 3480, 2980, 2930, 1730, 1710, 1650, 1620, 1460,  
1380, 1160, 1140  $\text{cm}^{-1}$

(10) <sup>1</sup>H Nuclear magnetic resonance spectrum:

(500 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$ (ppm):

35

15.0 (1H, br s), 7.76 (1H, d, 5), 5.97 (1H, d, 5), 5.74 (1H, br s),

4.22 (1H, dd, 10.5, 9.5), 4.13 (1H, dd, 10.5, 4.5), 3.20 - 3.13 (2H, m),  
2.63 (1H, m), 2.53 (1H, dd, 13, 10), 2.18 (1H, m), 2.12 (3H, br s), 2.02  
1H, m), 1.85 (1H, s), 1.80 (1H, m), 1.53 (3H, s), 1.57 - 1.47 (2H, m),  
1.37 (1H, m), 1.27 (1H, m), 1.27 (3H, s), 1.16 (3H, s), 1.12 (1H, dd,  
12, 14), 0.82 (3H, t, 7), 0.68 (3H, d, 6.5).

5 (11)  $^{13}\text{C}$  Nuclear magnetic resonance spectrum:

(125 MHz, CDCl<sub>3</sub>) δ(ppm):  
217.4 (s), 209.3 (s), 173.5 (d), 170.9 (s), 167.5 (s), 153.5 (s),  
119.7 (d), 102.7 (d), 78.1 (s), 69.6 (t), 68.9 (s), 50.5 (d), 50.2 (s),  
10 48.4 (t), 46.4 (t), 45.7 (d), 41.9 (t), 41.5 (d), 41.3 (d), 31.0 (q),  
30.1 (d), 24.6 (q), 22.5 (t), 21.3 (q), 20.6 (q), 19.1 (q), 13.4 (q).

15 2. A process for production of the WF00144 substance or its salt, which comprises culturing a WF00144 substance-producing microorganism in a nutrient medium and recovering the WF00144 substance or its salt from the resultant cultured broth.

3. Biological pure culture of *Phoma* sp. No. 00144 (FERM BP-6360).

20 4. A pharmaceutical composition containing the WF00144 substance or pharmaceutically acceptable salt thereof.

5. A use of the WF00144 substance as a medicament.

25 6. A method for treating or preventing diabetes which comprises administrating the WF00144 substance to human or animal.

7. Use of the WF00144 substance for the manufacture of a medicament for therapeutic treatment or prevention of diabetes in human or animal.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/JP 99/02707

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12P1/00 C12P1/02 C12P7/40 C07C53/00 C12N1/14				
According to International Patent Classification (IPC) or to both national classification and IPC				
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12P C07C C12N				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
Category	Citation of document, with indication, where appropriate, of the relevant passages			Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 123, no. 15, 9 October 1995 (1995-10-09) Columbus, Ohio, US; abstract no. 188618, YAMADA, MASASHI ET AL: "Aldose reductase inhibitors containing 2-hydroxy-p-benzoquinones" XP002110276 abstract & JP 07 149631 A (NIPPON KOKAN KK, JAPAN) 1993 ----			1-7
A,P	GB 2 323 845 A (MERCK & CO. INC.) 7 October 1998 (1998-10-07) the whole document -----			1-7
<input type="checkbox"/> Further documents are listed in the continuation of box C.		<input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed				
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family				
Date of the actual completion of the international search		Date of mailing of the international search report		
26 July 1999		09/08/1999		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl Fax: (+31-70) 340-3016		Authorized officer  Douschan, K		

# INTERNATIONAL SEARCH REPORT

## Information on patent family members

International Application No

PCT/JP 99/02707

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 7149631	A 13-06-1995	NONE	
GB 2323845	A 07-10-1998	NONE	